

(11) Publication number : **0 519 728 A2**

(12) **EUROPEAN PATENT APPLICATION**

(21) Application number : **92305627.9**

(51) Int. Cl.<sup>5</sup> : **C12P 21/08, G01N 33/577,  
G01N 33/68, // C07K3/18**

(22) Date of filing : **19.06.92**

(30) Priority : **21.06.91 JP 177236/91**

(43) Date of publication of application :  
**23.12.92 Bulletin 92/52**

(84) Designated Contracting States :  
**AT BE CH DE DK ES FR GB IT LI NL SE**

(71) Applicant : **SNOW BRAND MILK PRODUCTS &  
CO., LTD.**  
**1-1, Naebo-cho 6-chome Higashi-ku  
Sapporo-shi Hokkaido 065 (JP)**

(72) Inventor : **Higashio, Kanji  
1769-10, Yamada  
Kawagoe-shi, Saitama (JP)**  
Inventor : **Itagaki, Yasuharu  
1-27, Minami IIjo Nishi 18-chome  
Chuo-ku, Sapporo-shi (JP)**  
Inventor : **Ohgaki, Fumiko  
8-11, Yamato 1-chome  
Utsunomiya-shi, Tochigi (JP)**

(74) Representative : **Davies, Jonathan Mark  
Reddie & Grose 16 Theobalds Road  
London WC1X 8PL (GB)**

(54) **Anti-TCF-II monoclonal antibodies and method for the measurement of TCF-II by applying the antibodies.**

(57) Disclosed are monoclonal antibodies having specific affinity to an anti-tumor protein, TCF-II, derived from human fibroblasts. The monoclonal antibodies have a molecular weight of approximately 150,000 and belong to a subclass of IgG<sub>1</sub>, with N-terminal amino acid sequence of the L chain identified. The monoclonal antibodies can be used for the purification of TCF-II and for the diagnosis of liver disease by measuring the content of TCF-II in the plasma by the aid of the monoclonal antibodies.

**EP 0 519 728 A2**

**BACKGROUND OF THE INVENTION****1. Field of the Invention**

5 The present invention relates to novel monoclonal antibodies to a human anti-tumor protein, termed TCF-II, and a method for the measurement of TCF-II by employing the monoclonal antibodies.

**2. Description of the Related Art**

10  $\beta$ -Interferon is well known as a tumor cytotoxic factor, which is produced by human fibroblasts. Other substances produced by the fibroblasts are disclosed, respectively, in Japanese Patent Laid-open Publication (ko-kai) Nos. 58-146,293, 61-33,120, 61-1,872, 62-103,021 and 64-10,998. In the process of the investigation of anti-tumor proteins derived from the human fibroblasts, the present inventors have found a novel anti-tumor protein different from those having so far been reported and succeeded in the cloning of a cDNA encoding this protein. Further, they have determined all the primary amino acid sequence of this protein and confirmed its utility. This novel anti-tumor protein and its gene are disclosed in WO90/10651, and the novel anti-tumor protein is termed as TCF-II. All the primary amino acid sequences of the anti-tumor protein, TCF-II, which is deduced from its cDNA, is indicated in Table 1.

It is further confirmed that the TCF-II has a strong anti-tumor activity and a growth stimulating activity for normal cells and that it is a kind of families of hepatocyte growth factors, HGFs, which are potent growth stimulators for hepatocytes. The TCF-II has the molecular weight (MW) of  $78,000 \pm 2,000$  or  $74,000 \pm 2,000$ , on SDS-polyacrylamide gel electrophoresis under non-reducing conditions. Under the non-reducing conditions, it separates into three polypeptide chains: A-chain, as a common band, with the MW of  $52,000 \pm 2,000$ ; B-chain with the MW of  $30,000 \pm 2,000$ ; and C-chain with the MW of  $26,000 \pm 2,000$ . The N-terminus of the A-chain is blocked. The B- and C-chains have the same N-terminal amino acid sequence. The TCF-II essentially has a heterodimer structure composed of the A-chain and B-chain or C-chain.

It is further to be noted that neither monoclonal nor polyclonal antibodies to the TCF-II have been produced.

With attention paid to the utility of the TCF-II, the present inventors have investigated the utilization of the TCF-II as a anti-tumor agent or a diagnostic marker. In order to quantitate the TCF-II, there has so far been employed only biological assay method that utilizes the cytotoxic effect on tumor cells. Immunological assay is currently taking the main place due to accuracy of the determination of such a substance as being contained in a minute amount, so that strong demands have been made to develop an antibody capable of being employed for the measurement of the TCF-II. It is further noted that, although the TCF-II can be purified by repeating classical gel filtration and adsorptive chromatographies, the use of the antibody can remarkably improve the efficiency of purifying and recovering the TCF-II by taking advantage of affinity chromatography using such antibodies.

**SUMMARY OF THE INVENTION**

40 Therefore, the present invention has the object to provide monoclonal antibodies having high specificity and high affinity to TCF-II.

Another object of the present invention is to provide a method for measuring the TCF-II by employing the monoclonal antibodies.

**BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 shows standard curves for measuring TCF-II, being obtainable in various combination of the monoclonal antibodies.

Fig. 2 shows the results of recovery of the TCF-II added to the plasma and specificity of the monoclonal antibodies, used in the measurement system, to the TCF-II.

Fig. 3 shows the results of measurement for the contents of TCF-II in the plasma samples from patients with liver diseases.

Fig. 4 shows changes in the TCF-II levels in the patients with acute hepatitis at acute stage and at the recovery stage.

**DESCRIPTION OF THE PREFERRED EMBODIMENTS**

The monoclonal antibodies according to the present invention indicate specific affinity to human anti-tumor

protein termed TCF-II. The antibodies have a molecular weight of about 150,000 and belong to IgG<sub>1</sub> subclass. Further, each monoclonal antibody has an N-terminal amino acid sequence of its L chain defined by either one of the amino acid sequences (1) to (3), inclusive, as follows:

5 Amino acid sequence (1):

Asp-Val-Val-Leu-Thr-Gln-Ser-Pro-Ala-

1 2 3 4 5 6 7 8 9

10

Thr-Leu-Ser-Val-Thr-Pro-Gly-Asp-Ser-

10 11 12 13 14 15 16 17 18

15

Val

19

20 Amino acid sequence (2):

Asp-Ser-Val-Leu-Thr-Gln-Ser-Pro-Ala-

1 2 3 4 5 6 7 8 9

25

Ser-Leu-Ala-Val-Ser-Leu-Gly-Gln-Arg-

10 11 12 13 14 15 16 17 18

30

Ala-Thr-Ile-Ser

19 20 21 22

35

Amino acid sequence (3):

Asp-Ile-Val-Leu-Thr-Gln-Ser-Pro-Val-

1 2 3 4 5 6 7 8 9

40

Thr-Leu-Ser-Val-Thr-Pro-Gly-Gly-Ser-

10 11 12 13 14 15 16 17 18

45

Val

19

50

The monoclonal antibody according to the present invention can be prepared in the manner as will be described hereinafter. The monoclonal antibody can be prepared in accordance with conventional procedures by employing TCF-II as an antigen. The TCF-II can be produced from human fibroblasts according to the procedures as described in WO90/10651 or from microorganisms or other cells by recombinant technique on the basis of the cDNA sequence disclosed in WO90/10651. Further, there also can be employed synthetic peptides synthesized on the basis of the amino acid sequence of the TCF-II or peptides derived from partially decomposed TCF-II. It is also to be noted that these antigens may not necessarily be purified homogeneously. The

antigen is applied to immunize mammalian animals *in vivo* or lymphocytes (splenocytes) *in vitro*, and then the splenocytes are fused with myeloma cells derived from a mammalian animal. The fusion suspension is distributed into 96-well plates and cultured in medium containing HAT (hypoxanthine, amino protein, and thymidine). After 7-10 days, the plates are examined for the growth of hybridomas. The supernatant removed from these cells was screened for the presence of anti-TCF-II antibodies by ELISA. The hybrid cells which produce the desired antibody are cloned by limiting dilution and the clones are assayed again. The established hybridomas may be cultured in flasks or grown in mice to produce the objective antibodies.

In employing the mammalian animals in the formation of the hybridomas, it is common to use a small animal such as mouse or rat although there may be employed any mammalian animals.

Immunization may be carried out by diluting the TCF-II with physiological saline to appropriate concentrations, administering the small animals such as mice or rats with a given quantity of the resulting solution through intravenous or intraperitoneal route two to five times at every two to twenty days. An adjuvant is administered, if needed or when desired, in combination with a TCF-II antigen. The animals are then sacrificed in the third day after final immunization and the spleens are taken out from the animals. The splenocytes are fused with a mouse myeloma cell line, for example, p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP2/0, FO, P3x63Ag8.653, S194 and so on. Rat-derived cells may include cell lines such as R-210. A humanized antibody may be produced by immunizing human B lymphocytes *in vitro* and employing human myeloma cell lines or human B cell lines transformed by EB viruses as a parent cell line.

The fusion of the splenocytes with the myeloma cells may be carried out in conventional manner, as disclosed, for example, in Koehler, G., et al.: Nature, Vol. 256, 11 495-497, 1975. An electric pulse method may also be employed. The splenocytes are mixed with the myeloma cells in a conventional ratio, and the mixture is incubated for fusion in a serum-free medium which is conventionally employed for cell culture, to which polyethylene glycol has been added. The fusion suspension is distributed into 96-well plates and cultured in HAT medium containing FCS to select fused cells.

For the screening of the cells capable of producing anti-TCF-II antibodies, there may be employed procedures as have been conventionally employed for detection of antibodies, such as ELISA, plaque, ouchterlony, aggregation or the like. Among them, the ELISA method using purified TCF-II can select the desired antibody-producing cells with comparatively easy and high accuracy.

The hybridomas selected in the manner as described above can be sub-cultured in conventional manner and may be frozen for storage as needed. The hybridomas may be incubated in conventional manner and transplanted peritoneally into mice. The ascites containing anti-TCF-II antibodies are recovered from the mice and the antibodies in the ascites are purified by conventional method such as salting-out, gel filtration, affinity chromatography or the like.

The resulting antibody is found to react specifically with the TCF-II and can be employed for measurement and purification of the TCF-II. In using the antibody for the measurement of the TCF-II, the antibody is labelled with an isotope or an enzyme and can be employed as an antibody specific to the TCF-II in the measurement system which is known as radioimmunoassay (RIA) or enzyme immunoassay (EIA). Since each antibody in the present invention recognizes the different sites in the molecule of the TCF-II, the antibodies can be employed for sandwich immunoassay. The use of this assay system makes it possible to easily measure the amount of the TCF-II antigen in samples such as blood, urine and so on as well as a culture solution.

The TCF-II can readily be purified by means of immunoprecipitation or affinity chromatography prepared by immobilizing the antibody on a carrier such as Affigel 10 (Biorad).

The present invention will then be described more in detail by way of examples.

#### Example 1

##### Purification of TCF-II for Antigen

Purified TCF-II was prepared by incubating cells in accordance with the method as disclosed in WO90/10651 and the purification method as disclosed by Higashio, K. et al. (B.B.R.C., vol. 170, pp. 397-404, 1990).

A total number of  $3 \times 10^6$  cells of human fibroblasts, IMR-90 (ATCC CCL 186), was seeded in a roller bottle (one liter; Corning) containing 100 ml of DMEM with 10% calf serum (Hyclone). The cells were cultured by rotating the roller bottle at 0.5 to 2 rpm for seven days. The cells were then trypsinized when the total cell numbers reached  $1 \times 10^7$ . The cells were suspended in 250 ml of DMEM with 10% calf serum and allowed to settle to the bottom of the bottle. Ceramic pieces (3.5 to 5.0 mesh; Toshiba Ceramics Co., Ltd.) were autoclaved and 100 grams of the autoclaved ceramic pieces were added to the cell suspension in the roller bottle as the cell matrices; static culture was continued for 24 hours. After the static culture at 37° C for 24 hours, the roller bottle

was supplemented with 250 ml of the same medium and further incubated for 7 days. The culture medium was replaced with 500 ml of DMEM supplemented with 5% calf serum in every 7 to 10 days. The conditioned medium was collected during two months at a rate of four liters from every roller bottle.

The activity of the TCF-II in the collected conditioned medium was found to be 32 units per milliliter.

Seventy five liters of the culture medium were concentrated by treatment with ultrafiltration module (MW 6,000 cuts; Asahi Chemical Industries Co., Ltd.) in accordance with the method as described in Patent Publication No. 90/10651 (WO90/10651). The TCF-II in the concentrate was then purified by a four-step chromatography consisting of CM Sephadex C-50 (Pharmacia), ConA Sephadex (Pharmacia), Mono S column (Pharmacia) and Heparin Sepharose (Pharmacia), thereby yielding the purified TCF-II having the specific activity of 5,248,000 units per mg.

## Example 2

### Preparation of Hybridoma Producing Anti-TCF-II Antibody

The purified TCF-II obtained in Example 1 was dissolved in PBS at a concentration of 10 µg per 100 µl, and BALB/c mice were immunized with the resulting solution at every two weeks. At the first and second immunization, a mixture of Freund's complete adjuvant and the TCF-II solution (1 : 1) was administered to the mice. In the third day after final immunization, the spleens of the mice were taken out and B lymphocytes were isolated from the spleens. Then, the resulting B lymphocytes were fused with mouse myeloma cells, P3x63-AG8.653, in accordance with the method as described by Koehler, G., et al. (Nature: vol. 256, pp. 495-497, 1975). The fused cells were cultured in a HAT culture medium. In order to select hybridomas producing the antibody specific to the TCF-II, a supernatant of hybridoma culture was measured for its antibody specific to the TCF-II by means of solid phase ELISA using a microplate coated with the TCF-II. The hybridoma which had produced the objective antibody was cloned five or six times by the limiting dilution technique, while the quantity of the antibody produced by the cloned hybridoma was measured at every time by means of the ELISA.

Then, the clones which have high productivities of the antibodies were selected from the cloned hybridomas.

## Example 3

### Production of Monoclonal Antibody

An amount of  $1 \times 10^6$  cells of the strain producing a high amount of the antibody, obtained in Example 2, was intraperitoneally transplanted into Balb/c mice which were primed with pristane (Aldrich Chemicals, Inc.). In two weeks after transplantation, the accumulated ascites were collected, thereby producing the ascites containing the monoclonal antibodies according to the present invention. The antibody in the ascites was purified by means of Affigel Protein A Sepharose® (BioRad) chromatography in accordance with its manual. In other words, the ascites was diluted with the equal amount of a binding buffer (BioRad), and the resulting dilution was charged onto a protein A column, followed by washing the column with a sufficient amount of the binding buffer. The antibody, IgG, was eluted with an elution buffer (BioRad) and, after neutralization, the resulting eluate was then dialyzed against water, followed by lyophilization. The purified antibody was then subjected to SDS-PAGE electrophoresis to check its purity and it was found that the purified antibody migrates as a homogeneous band having a molecular weight of approximately 150,000.

## Example 4

### Selection of Monoclonal Antibody Having High Affinity to TCF-II

The antibodies obtained in Example 3 were dissolved in PBS and the concentration of the protein was determined by the Lowry method. Then, each of the antibodies was dissolved in PBS to give the same concentration. The resulting antibody solution was diluted by means of stepwise dilution, and the resulting antibody dilutions were measured by solid phase ELISA, as described in Example 2 above, to select the monoclonal antibody that can react with the TCF-II even at high dilution rates. As a result, three antibodies were selected and they were referred to as P5A8, P2D6 and P1C8. The strains producing the antibodies P5A8, P2D6 and P1C8 were referred to as P5A8 strain, P2D6 strain and P1C8 strain and deposited with under Deposit Nos. BP-3820, BP-3821 and BP-3822, respectively.

**Example 5****Analysis of Subclass of Antibody**

- 5 The subclass of each antibody selected in Example 4 was analyzed by an immunoglobulin subclass analysis kit (Funakoshi) in accordance with protocol as instructions as given on the kit. The analysis results are shown in Table 2.

T A B L E 2

Name of Antibody	IgG <sub>1</sub>	IgG <sub>2a</sub>	IgG <sub>2b</sub>	IgG <sub>3</sub>	IgA	IgM
15 P5A8	+	-	-	-	-	-
P2D6	+	-	-	-	-	-
20 P1C8	+	-	-	-	-	-

As is apparent from Table 2, all the monoclonal antibodies were found positive to IgG<sub>1</sub> only, so that all the three monoclonal antibodies were confirmed that they belong to a subclass of IgG<sub>1</sub>.

**Example 6****Determination of N-Terminal Amino Acid Sequence of Antibody**

- 30 It is well known that antibodies are proteins having the structure consisting of two chains, i.e. light and heavy chains. The two chains are classified into a heavy chain (a H chain) having a larger molecular weight and a light chain (an L chain) having a smaller molecular weight, and each of the chains of the monoclonal antibodies can be defined by their N-terminal amino acid sequences.

The N-terminal amino acid sequence of each antibody was determined in the manner as will be described hereinafter.

- 35 The purified antibody selected in Example 4 was dissolved at a final concentration of 2 µg/µl in 150 µl of SDS-PAGE buffer solution (a 10 mM Tris-hydrochloride buffer solution (pH 8.0) containing 1 mM of EDTA, 25% SDS, 0.01% BPB, 10% mercaptoethanol, and 10% glycerol). After the solution was heated at 100° C for 3 minutes, the antibody solution was centrifuged at 15,000 rpm for 3 minutes. The resulting supernatant was subjected to electrophoresis using a 10% SDS polyacrylamide gel. The H and L chains of the antibody were electrically transferred to a PVDF (polyvinylidene difluoride) membrane by the wester blotting method. The PVDF membrane with protein was stained with a Coomassie Brilliant Blue and the membrane with bands corresponding to the objective H and L chains were cut out. The cut membranes were introduced directly into vapor-phase protein sequencer (ABI) and subjected automatically to coupling, cleavage and conversion in accordance with the protocols, thereby yielding phenyl thiohydantoin (PTH) amino acid which in turn was dissolved in a 20% acetonitrile. The resulting solution was then applied to reverse-phase high-pressure liquid chromatography (ABI; C-18 column) and each PTH-amino acid was identified by comparing the retention time thereof with that of a standard PTH-amino acid. Although neither of the H chains of the three antibodies could be determined because their N-terminal amino acids were blocked, the L chains of the three antibodies were found to having the N-terminal amino acid sequences as follows:

50

55

## Antibody P5A8:

5            Asp-Val-Val-Leu-Thr-Gln-Ser-Pro-Ala-  
              1    2    3    4    5    6    7    8    9  
              Thr-Leu-Ser-Val-Thr-Pro-Gly-Asp-Ser-  
 10           10   11   12   13   14   15   16   17   18  
              Val  
 15           19

## Antibody P2D6:

20           Asp-Ser-Val-Leu-Thr-Gln-Ser-Pro-Ala-  
              1    2    3    4    5    6    7    8    9  
              Ser-Leu-Ala-Val-Ser-Leu-Gly-Gln-Arg-  
 25           10   11   12   13   14   15   16   17   18  
              Ala-Thr-Ile-Ser  
 30           19   20   21   22

## Antibody P1C8:

35           Asp-Ile-Val-Leu-Thr-Gln-Ser-Pro-Val-  
              1    2    3    4    5    6    7    8    9  
 40           Thr-Leu-Ser-Val-Thr-Pro-Gly-Gly-Ser-  
              10   11   12   13   14   15   16   17   18  
 45           Val  
              19

## Example 7

50

## Measurement for TCF-II by ELISA

55           The best combination of the antibodies for sandwich ELISA was selected by using three antibodies P5A8, P2D6 and P1C8 as solid phase and labelled antibodies. The solid phase antibody of each antibody was prepared by dissolving the antibody at a final concentration of 10 µg per ml in a 0.1M sodium bicarbonate solution, pouring 100 µl of the solution into each well in 96-well microplates (NunC), allowing the microplates to stand overnight at room temperature. Subsequently, each well was filled with PBS containing a 1% BSA (bovine serum albumin), and then the microplates were allowed to stand at room temperature for one hour to block the

residual binding sites on the plates; the microplates were washed three times with a washing buffer (PBS containing 0.05% Tween 20). On the other hand, each monoclonal antibody was labelled with peroxidase in accordance with the method devised by Ishikawa et al. (J. Immunoassay, vol. 4, pp. 209-327, 1983).

Samples of the TCF-II were prepared by diluting the TCF-II solution with a diluting buffer (PBS containing 0.0.1% BSA and 0.05% Tween® 20) and 100 µl of the sample was added to each well of the microplates. The microplates were allowed to stand at 37° C for 3 hours, followed by washing each well three times with the washing buffer, and adding 100 µl of the labelled antibody diluted 200- to 400-fold with the diluting buffer to each well. After the labelled antibody was added, the microplates were allowed to stand at 37° C for 2 hours and washed three times with the washing buffer, followed by adding 100 µl of a substrate solution (a 0.1 M citrate-phosphate buffer (pH 4.5) containing 0.4mg/ml of orthophenylenediamine hydrochloride and 0.006% hydrogen peroxide) to each well in the microplates. After the microplates were allowed to stand at 37° C in dark place for 30 minutes, 50 µl of 6N sulfuric acid was added to each well to stop enzymatic reaction and absorbency of each well at 492 nm was measured by microplate spectrophotometer (Corona).

The combinations between the solid phase antibodies and the labelled antibodies are as shown in Table 3.

T A B L E 3

Solid Phase Antibody	Labelled Antibody
P1C8	P2D6
P5A8	P2D6
P5A8	P1C8

It can be noted that Fig. 1 shows the standard curves for measuring TCF-II, obtained at different combinations of the monoclonal antibodies. In Fig. 1, reference symbol "■-■" denotes a combination of the solid phase monoclonal antibody P1C8 and the labelled monoclonal antibody P2D6; reference symbol "○-○" denotes a combination of the solid phase monoclonal antibody P5A8 and the labelled monoclonal antibody P2D6; and reference symbol "□-□" denotes a combination of the solid phase monoclonal antibody P5A8 and the labelled monoclonal antibody P1C8. It can be found as a result that the measurement by the combination of the monoclonal antibodies P1C8 and P2D6 as well as that of the monoclonal antibodies P5A8 and P2D6 gave the good results.

It is further to be noted as apparent that each of the monoclonal antibodies P1C8, P5A8 and P2D6 has the property of recognizing epitopes which are different from each other.

#### Example 8

##### Determination of TCF-II in Human Plasma

The following tests were carried out using the combination of the solid phase monoclonal antibody P1C8 and the labelled monoclonal antibody P2D6.

##### 1. Standard curve of TCF-II and recovery of added TCF-II in Plasma

As the solid phase antibody, the monoclonal antibody P1C8 was dissolved at a final concentration of 10 µg/ml in a 0.1 M sodium bicarbonate solution, and 100 µl of the solution was added to each well in 96-well microplates (NunC), followed by allowing the microplates to stand overnight at room temperature. The wells in the microplates were then filled with a solution which had been prepared by diluting Block Ace® (Snow Brand) twofold with distilled water in accordance with the procedures as described by Shinmoto et al. (Reports of Snow Brand Research Laboratories, vol. 88, pp. 45-51, 1989). The blocking was implemented by allowing the microplate to stand at room temperature for 1 hour, and the microplates were washed three times with a washing buffer (PBS containing 0.05% Tween® 20).

Then, the TCF-II samples having various concentrations were prepared by diluting the TCF-II solution with a diluting buffer (PBS containing 50% Block Ace® and 0.1% Tween® 20). To examine the recovery of the TCF-



II from human plasma, samples of plasma containing exogenous TCF-II were prepared by adding the TCF-II having the same concentration series to human plasma. As a negative control, plasminogen, which is assumed to be high in structural homology to TCF-II (Nakamura et al.: Nature, vol. 34, pp. 440-443, 1989), was prepared by diluting the plasminogen solution with the same diluting buffer. A volume of 50  $\mu$ l of the first reaction buffer (0.2M Tris-Hydrochloride buffer containing 50% Block Ace®, 0.2 M NaCl, 0.1% Tween® 20, 0.2% CHAPS, 20 mM benzamidine hydrochloride and 10 mM EDTA) were applied to each well in the microplates, and then a volume of 50  $\mu$ l of each sample was applied to each well con 50  $\mu$ l of the first buffer. The microplates were allowed to stand at 37° C for 3 hours, followed by washing three times with the washing buffer.

For the dilution of the labelled antibody, there was employed a 0.1 M phosphate buffer, pH 7, containing 1/10 Block Ace®, 0.15 M NaCl, 0.1% Tween® 20, 4% rat serum and 500  $\mu$ g/ml of mouse IgG. To each well in the microplates was added 100  $\mu$ l of a 400-fold dilution of the antibody P2D6 labelled with peroxidase. The microplates were allowed to stand at 37° C for 2 hours, followed by washing three times with the washing buffer and adding 100  $\mu$ l of a substrate solution (a 0.1 M citrate-phosphate buffer, pH 4.5, containing 0.4 mg/ml of orthphenylenediamine hydrochloride and 0.006% hydrogen peroxide). The resulting microplates were then allowed to stand in dark place at 37° C for 30 minutes and 50  $\mu$ l of 6 N sulfuric acid was added to each well in the microplates to cease the enzymatic reaction. Then, the absorbance at 492 nm of each well in the microplates was measured by the microplate spectrophotometer (corona).

Fig. 2 shows the results of recovery of the TCF-II from the plasma and the specificity of the antibodies used in the assay system to the TCF-II. In Fig. 2, reference symbol "●-●" indicates the plasma samples to which the TCF-II was added; reference symbol "X-X" indicates standard TCF-II; reference symbol "◆-◆" indicates plasminogen.

As is apparent from Fig. 2, it is found that the sample with TCF-II added to the plasma indicates the line parallel to the standard curve of the TCF-II and it is thus confirmed that the whole amount of the TCF-II in the plasma has been recovered and that the plasminogen did not react at all in the measurement system.

## 2. Determination of TCF-II in the plasma samples from patients with liver diseases

The levels of the TCF-II in the plasma samples from 45 patients with liver diseases were determined by means of the system as described in item 1 above. The liver diseases include fulminant hepatitis, acute hepatitis, chronic active hepatitis, chronic inactive hepatitis, compensated liver cirrhosis, decompensated liver cirrhosis, and hepatocellular carcinoma.

Fig. 3 indicates the results of determination of the TCF-II in the plasma from the patients with liver diseases. In Fig. 3, reference symbol "●" indicates a patient with chronic active hepatitis; reference symbol "□-□" indicates an average and the range of  $\pm 1$ SD; reference symbol "□" indicates the range of the TCF-II for normal subjects. Fig. 3 indicates a normal control obtained from 21 healthy volunteers. Further, in Fig. 3, abbreviation "FH" means fulminant hepatitis; "AH" means acute hepatitis; "CH" means chronic hepatitis (inactive & active); "cLC" means compensated liver cirrhosis; "dLC" means a decompensated liver cirrhosis; and "HCC" means hepatocellular carcinoma. It is apparent from Fig. 3 that the plasma collected from the patients with liver diseases indicated a higher average concentration of the TCF-II than those collected from the normal persons.

## 3. Measurement for TCF-II in the plasma samples from the patients with acute hepatitis in the acute stage and in the recovery stage

The levels of the TCF-II in the plasma samples from 8 patients with acute hepatitis were measured. The results of measurement are shown in Fig. 4. The results in Fig. 4 reveal that, except for one case, the levels of the TCF-II are reduced in the recovery stage of the acute hepatitis for the rest of the cases, so that it is recognized that the measurement of the TCF-II levels can serve as an indicator representing changes of the pathema of a patient with acute hepatitis.

As described hereinabove, the present invention can provide the monoclonal antibody to TCF-II, and the monoclonal antibody to the TCF-II is useful for the purification and determination of the TCF-II. The method for the determination of the TCF-II using the monoclonal antibodies according to the present invention can permit an accurate diagnosis of liver diseases or their pathemas by using an extremely small amount of samples.

## SEQUENCE LISTING

5

Information for Seq. No: 1

Length: 19

10

Type: amino acids

Topology: linear

15

Molecular type: peptide

Sequence:

20

Asp Val Val Leu Thr Gln Ser Pro Ala Thr Leu Ser Val Thr Pro Gly

1

5

10

15

Asp Ser Val

25

19

30

Information for Seq. No:2

Length: 22

35

Type: amino acids

Topology: linear

Molecular type: peptide

40

Sequence:

45

Asp Ser Val Leu Thr Gln Ser Pro Ala Ser Leu Ala Val Ser Leu Gly

1

5

10

15

Gln Arg Ala Thr Ile Ser

20

22

50

55

Information for Seq. No: 3

Length: 19

5 Type: amino acids

Topology: linear

10 Molecular type: peptide

Sequence:

15 Asp Ile Val Leu Thr Gln Ser Pro Val Thr Leu Ser Val Thr Pro Gly  
 1 5 10 15  
 Gly Ser Val  
 20 19

Information for Seq. No:4

25 Length: 723

Type: amino acids

30 Topology: linear

Molecular type: protein

Sequence:

35 Met Trp Val Thr Lys Leu Leu Pro Ala Leu Leu Leu Gln His Val Leu  
 1 5 10 15  
 40 Leu His Leu Leu Leu Leu Pro Ile Ala Ile Pro Tyr Ala Glu Gly Gln  
 20 25 30  
 Arg Lys Arg Arg Asn Thr Ile His Glu Phe Lys Lys Ser Ala Lys Thr  
 45 35 40 45  
 Thr Leu Ile Lys Ile Asp Pro Ala Leu Lys Ile Lys Thr Lys Lys Val  
 50 50 55 60  
 Asn Thr Ala Asp Gln Cys Ala Asn Arg Cys Thr Arg Asn Lys Gly Leu  
 65 70 75 80

55

	Pro Phe Thr Cys Lys Ala Phe Val Phe Asp Lys Ala Arg Lys Gln Cys		
	85	90	95
5	Leu Trp Phe Pro Phe Asn Ser Met Ser Ser Gly Val Lys Lys Glu Phe		
	100	105	110
10	Gly His Glu Phe Asp Leu Tyr Glu Asn Lys Asp Tyr Ile Arg Asn Cys		
	115	120	125
	Ile Ile Gly Lys Gly Arg Ser Tyr Lys Gly Thr Val Ser Ile Thr Lys		
15	130	135	140
	Ser Gly Ile Lys Cys Gln Pro Trp Ser Ser Met Ile Pro His Glu His		
20	145	150	155
	Ser Tyr Arg Gly Lys Asp Leu Gln Glu Asn Tyr Cys Arg Asn Pro Arg		
	165	170	175
25	Gly Glu Glu Gly Gly Pro Trp Cys Phe Thr Ser Asn Pro Glu Val Arg		
	180	185	190
30	Tyr Glu Val Cys Asp Ile Pro Gln Cys Ser Glu Val Glu Cys Met Thr		
	195	200	205
	Cys Asn Gly Glu Ser Tyr Arg Gly Leu Met Asp His Thr Glu Ser Gly		
35	210	215	220
	Lys Ile Cys Gln Arg Trp Asp His Gln Thr Pro His Arg His Lys Phe		
40	225	230	235
	Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe Asp Asp Asn Tyr Cys Arg		
	245	250	255
45	Asn Pro Asp Gly Gln Pro Arg Pro Trp Cys Tyr Thr Leu Asp Pro His		
	260	265	270
50	Thr Arg Trp Glu Tyr Cys Ala Ile Lys Thr Cys Ala Asp Asn Thr Met		
	275	280	285
	Asn Asp Thr Asp Val Pro Leu Glu Thr Thr Glu Cys Ile Gln Gly Gln		
55	290	295	300

Gly Glu Gly Tyr Arg Gly Thr Val Asn Thr Ile Trp Asn Gly Ile Pro  
 305                      310                      315                      320  
 5 Cys Gln Arg Trp Asp Ser Gln Tyr Pro His Glu His Asp Met Thr Pro  
                          325                      330                      335  
 10 Glu Asn Phe Lys Cys Lys Asp Leu Arg Glu Asn Tyr Cys Arg Asn Pro  
                          340                      345                      350  
 Asp Gly Ser Glu Ser Pro Trp Cys Phe Thr Thr Asp Pro Asn Ile Arg  
 15                      355                      360                      365  
 Val Gly Tyr Cys Ser Gln Ile Pro Asn Cys Asp Met Ser His Gly Gln  
                          370                      375                      380  
 20 Asp Cys Tyr Arg Gly Asn Gly Lys Asn Tyr Met Gly Asn Leu Ser Gln  
 385                      390                      395                      400  
 25 Thr Arg Ser Gly Leu Thr Cys Ser Met Trp Asp Lys Asn Met Glu Asp  
                          405                      410                      415  
 Leu His Arg His Ile Phe Trp Glu Pro Asp Ala Ser Lys Leu Asn Glu  
 30                      420                      425                      430  
 Asn Tyr Cys Arg Asn Pro Asp Asp Asp Ala His Gly Pro Trp Cys Tyr  
 35                      435                      440                      445  
 Thr Gly Asn Pro Leu Ile Pro Trp Asp Tyr Cys Pro Ile Ser Arg Cys  
                          450                      455                      460  
 40 Glu Gly Asp Thr Thr Pro Thr Ile Val Asn Leu Asp His Pro Val Ile  
 465                      470                      475                      480  
 45 Ser Cys Ala Lys Thr Lys Gln Leu Arg Val Val Asn Gly Ile Pro Thr  
                          485                      490                      495  
 Arg Thr Asn Ile Gly Trp Met Val Ser Leu Arg Tyr Arg Asn Lys His  
 50                      500                      505                      510  
 Ile Cys Gly Gly Ser Leu Ile Lys Glu Ser Trp Val Leu Thr Ala Arg  
 55                      515                      520                      525

Gln Cys Phe Pro Ser Arg Asp Leu Lys Asp Tyr Glu Ala Trp Leu Gly  
 530 535 540  
 5 Ile His Asp Val His Gly Arg Gly Asp Glu Lys Cys Lys Gln Val Leu  
 545 550 555 560  
 10 Asn Val Ser Gln Leu Val Tyr Gly Pro Glu Gly Ser Asp Leu Val Leu  
 565 570 575  
 Met Lys Leu Ala Arg Pro Ala Val Leu Asp Asp Phe Val Ser Thr Ile  
 15 580 585 590  
 Asp Leu Pro Asn Tyr Gly Cys Thr Ile Pro Glu Lys Thr Ser Cys Ser  
 595 600 605  
 20 Val Tyr Gly Trp Gly Tyr Thr Gly Leu Ile Asn Tyr Asp Gly Leu Leu  
 610 615 620  
 25 Arg Val Ala His Leu Tyr Ile Met Gly Asn Glu Lys Cys Ser Gln His  
 625 630 635 640  
 30 His Arg Gly Lys Val Thr Leu Asn Glu Ser Glu Ile Cys Ala Gly Ala  
 645 650 655  
 Glu Lys Ile Gly Ser Gly Pro Cys Glu Gly Asp Tyr Gly Gly Pro Leu  
 35 660 665 670  
 Val Cys Glu Gln His Lys Met Arg Met Val Leu Gly Val Ile Val Pro  
 675 680 685  
 40 Gly Arg Gly Cys Ala Ile Pro Asn Arg Pro Gly Ile Phe Val Arg Val  
 690 695 700  
 45 Ala Tyr Tyr Ala Lys Trp Ile His Lys Ile Ile Leu Thr Tyr Lys Val  
 705 710 715 720  
 Pro Gln Ser  
 50 723

55

**Claims**

1. A monoclonal antibody having affinity specific to an human anti-carcinoma protein termed TCF-II and a

molecular weight of approximately 150,000 and belonging to subclass IgG<sub>1</sub>.

2. A monoclonal antibody as claimed in claim 1, wherein an N-terminal amino acid sequence of an L chain of the monoclonal antibody is defined by either one of the following amino acid sequences:

Amino acid sequence (1):

Asp-Val-Val-Leu-Thr-Gln-Ser-Pro-Ala-

1 2 3 4 5 6 7 8 9

Thr-Leu-Ser-Val-Thr-Pro-Gly-Asp-Ser-

10 11 12 13 14 15 16 17 18

Val

19

Amino acid sequence (2):

Asp-Ser-Val-Leu-Thr-Gln-Ser-Pro-Ala-

1 2 3 4 5 6 7 8 9

Ser-Leu-Ala-Val-Ser-Leu-Gly-Gln-Arg-

10 11 12 13 14 15 16 17 18

Ala-Thr-Ile-Ser

19 20 21 22

Amino acid sequence (3):

Asp-Ile-Val-Leu-Thr-Gln-Ser-Pro-Val-

1 2 3 4 5 6 7 8 9

Thr-Leu-Ser-Val-Thr-Pro-Gly-Gly-Ser-

10 11 12 13 14 15 16 17 18

Val

19

3. A method for measuring a human anti-tumor protein, TCF-II, by using a monoclonal antibody having affinity specific to a human anti-tumor protein termed TCF-II and a molecular weight of approximately 150,000 and belonging to subclass IgG<sub>1</sub>.

4. A method as claimed in claim 3, wherein said monoclonal antibody is defined by N-terminal amino acid sequence of L chain of the monoclonal antibodies comprising either one of the following amino acid se-

quences:

Amino acid sequence (1):

5 Asp-Val-Val-Leu-Thr-Gln-Ser-Pro-Ala-  
 1 2 3 4 5 6 7 8 9  
 10 Thr-Leu-Ser-Val-Thr-Pro-Gly-Asp-Ser-  
 10 11 12 13 14 15 16 17 18  
 Val  
 15 19

Amino acid sequence (2):

20 Asp-Ser-Val-Leu-Thr-Gln-Ser-Pro-Ala-  
 1 2 3 4 5 6 7 8 9  
 25 Ser-Leu-Ala-Val-Ser-Leu-Gly-Gln-Arg-  
 10 11 12 13 14 15 16 17 18  
 30 Ala-Thr-Ile-Ser  
 19 20 21 22

Amino acid sequence (3):

35 Asp-Ile-Val-Leu-Thr-Gln-Ser-Pro-Val-  
 1 2 3 4 5 6 7 8 9  
 40 Thr-Leu-Ser-Val-Thr-Pro-Gly-Gly-Ser-  
 10 11 12 13 14 15 16 17 18  
 45 Val  
 19

- 50 5. A method as claimed in claim 3 or 4, wherein said monoclonal antibody is in a combination of a solid phase monoclonal antibody and an enzyme-labelled monoclonal antibody.
6. Use of a monoclonal antibody as claimed in claim 1 for diagnosis of a liver disease.

55



TABLE 1: Sequence of Amino Acids of TCF-II

5	Met	Trp	Val	Thr	Lys	Leu	Leu	Pro	Ala	Leu	Leu	Leu	Gln	His
					5					10				
10	Val	Leu	Leu	His	Leu	Leu	Leu	Leu	Pro	Ile	Ala	Ile	Pro	Tyr
	15				20					25				
15	Ala	Glu	Gly	Gln	Arg	Lys	Arg	Arg	Asn	Thr	Ile	His	Glu	Phe
		30				35					40			
	Lys	Lys	Ser	Ala	Lys	Thr	Thr	Leu	Ile	Lys	Ile	Asp	Pro	Ala
20			45					50				55		
	Leu	Lys	Ile	Lys	Thr	Lys	Lys	Val	Asn	Thr	Ala	Asp	Gln	Cys
				60				65					70	
	Ala	Asn	Arg	Cys	Thr	Arg	Asn	Lys	Gly	Leu	Pro	Phe	Thr	Cys
					75					80				
30	Lys	Ala	Phe	Val	Phe	Asp	Lys	Ala	Arg	Lys	Gln	Cys	Leu	Trp
	85					90					95			
35	Phe	Pro	Phe	Asn	Ser	Met	Ser	Ser	Gly	Val	Lys	Lys	Glu	Phe
		100					105					110		
	Gly	His	Glu	Phe	Asp	Leu	Tyr	Glu	Asn	Lys	Asp	Tyr	Ile	Arg
40			115					120				125		
	Asn	Cys	Ile	Ile	Gly	Lys	Gly	Arg	Ser	Tyr	Lys	Gly	Thr	Val
45				130				135					140	
	Ser	Ile	Thr	Lys	Ser	Gly	Ile	Lys	Cys	Gln	Pro	Trp	Ser	Ser
50					145					150				

55

Met Ile Pro His Glu His Ser Tyr Arg Gly Lys Asp Leu Gln  
 5 155 160 165  
 Glu Asn Tyr Cys Arg Asn Pro Arg Gly Glu Glu Gly Gly Pro  
 170 175 180  
 10 Trp Cys Phe Thr Ser Asn Pro Glu Val Arg Tyr Glu Val Cys  
 185 190 195  
 15 Asp Ile Pro Gln Cys Ser Glu Val Glu Cys Met Thr Cys Asn  
 200 205 210  
 Gly Glu Ser Tyr Arg Gly Leu Met Asp His Thr Glu Ser Gly  
 20 215 220  
 Lys Ile Cys Gln Arg Trp Asp His Gln Thr Pro His Arg His  
 25 225 230 235  
 Lys Phe Leu Pro Glu Arg Tyr Pro Asp Lys Gly Phe Asp Asp  
 240 245 250  
 30 Asn Tyr Cys Arg Asn Pro Asp Gly Gln Pro Arg Pro Trp Cys  
 255 260 265  
 35 Tyr Thr Leu Asp Pro His Thr Arg Trp Glu Tyr Cys Ala Ile  
 270 275 280  
 40 Lys Thr Cys Ala Asp Asn Thr Met Asn Asp Thr Asp Val Pro  
 285 290  
 Leu Glu Thr Thr Glu Cys Ile Gln Gly Gln Gly Glu Gly Tyr  
 45 295 300 305  
 Arg Gly Thr Val Asn Thr Ile Trp Asn Gly Ile Pro Cys Gln  
 50 310 315 320

55

Arg Trp Asp Ser Gln Tyr Pro His Glu His Asp Met Thr Pro  
 5                   325                   330                   335  
 Glu Asn Phe Lys Cys Lys Asp Leu Arg Glu Asn Tyr Cys Arg  
 10                   340                   345                   350  
 Asn Pro Asp Gly Ser Glu Ser Pro Trp Cys Phe Thr Thr Asp  
                   355                   360  
 15 Pro Asn Ile Arg Val Gly Tyr Cys Ser Gln Ile Pro Asn Cys  
 365                   370                   375  
 20 Asp Met Ser His Gly Gln Asp Cys Tyr Arg Gly Asn Gly Lys  
 380                   385                   390  
 25 Asn Tyr Met Gly Asn Leu Ser Gln Thr Arg Ser Gly Leu Thr  
 395                   400                   405  
 Cys Ser Met Trp Asp Lys Asn Met Glu Asp Leu His Arg His  
 30                   410                   415                   420  
 Ile Phe Trp Glu Pro Asp Ala Ser Lys Leu Asn Glu Asn Tyr  
 35                   425                   430  
 Cys Arg Asn Pro Asp Asp Asp Ala His Gly Pro Trp Cys Tyr  
 435                   440                   445  
 40 Thr Gly Asn Pro Leu Ile Pro Trp Asp Tyr Cys Pro Ile Ser  
 450                   455                   460  
 45 Arg Cys Glu Gly Asp Thr Thr Pro Thr Ile Val Asn Leu Asp  
 465                   470                   475  
 50 His Pro Val Ile Ser Cys Ala Lys Thr Lys Gln Leu Arg Val  
 480                   485                   490  
 55

Val Asn Gly Ile Pro Thr Arg Thr Asn Ile Gly Trp Met Val  
 495 500  
 5 Ser Leu Arg Tyr Arg Asn Lys His Ile Cys Gly Gly Ser Leu  
 505 510 515  
 10 Ile Lys Glu Ser Trp Val Leu Thr Ala Arg Gln Cys Phe Pro  
 520 525 530  
 15 Ser Arg Asp Leu Lys Asp Tyr Glu Ala Trp Leu Gly Ile His  
 535 540 545  
 Asp Val His Gly Arg Gly Asp Glu Lys Cys Lys Gln Val Leu  
 20 550 555 560  
 Asn Val Ser Gln Leu Val Tyr Gly Pro Glu Gly Ser Asp Leu  
 25 565 570  
 Val Leu Met Lys Leu Ala Arg Pro Ala Val Leu Asp Asp Phe  
 575 580 585  
 30 Val Ser Thr Ile Asp Leu Pro Asn Tyr Gly Cys Thr Ile Pro  
 590 595 600  
 35 Glu Lys Thr Ser Cys Ser Val Tyr Gly Trp Gly Tyr Thr Gly  
 605 610 615  
 40 Leu Ile Asn Tyr Asp Gly Leu Leu Arg Val Ala His Leu Tyr  
 620 625 630  
 Ile Met Gly Asn Glu Lys Cys Ser Gln His His Arg Gly Lys  
 45 635 640  
 Val Thr Leu Asn Glu Ser Glu Ile Cys Ala Gly Ala Glu Lys  
 50 645 650 655  
 55

Ile Gly Ser Gly Pro Cys Glu Gly Asp Tyr Gly Gly Pro Leu  
660 665 670  
5 Val Cys Glu Gln His Lys Met Arg Met Val Leu Gly Val Ile  
675 680 685  
10 Val Pro Gly Arg Gly Cys Ala Ile Pro Asn Arg Pro Gly Ile  
690 695 700  
15 Phe Val Arg Val Ala Tyr Tyr Ala Lys Trp Ile His Lys Ile  
705 710  
Ile Leu Thr Tyr Lys Val Pro Gln Ser  
20 715 720 723  
25  
30  
35  
40  
45  
50  
55

Fig. 1

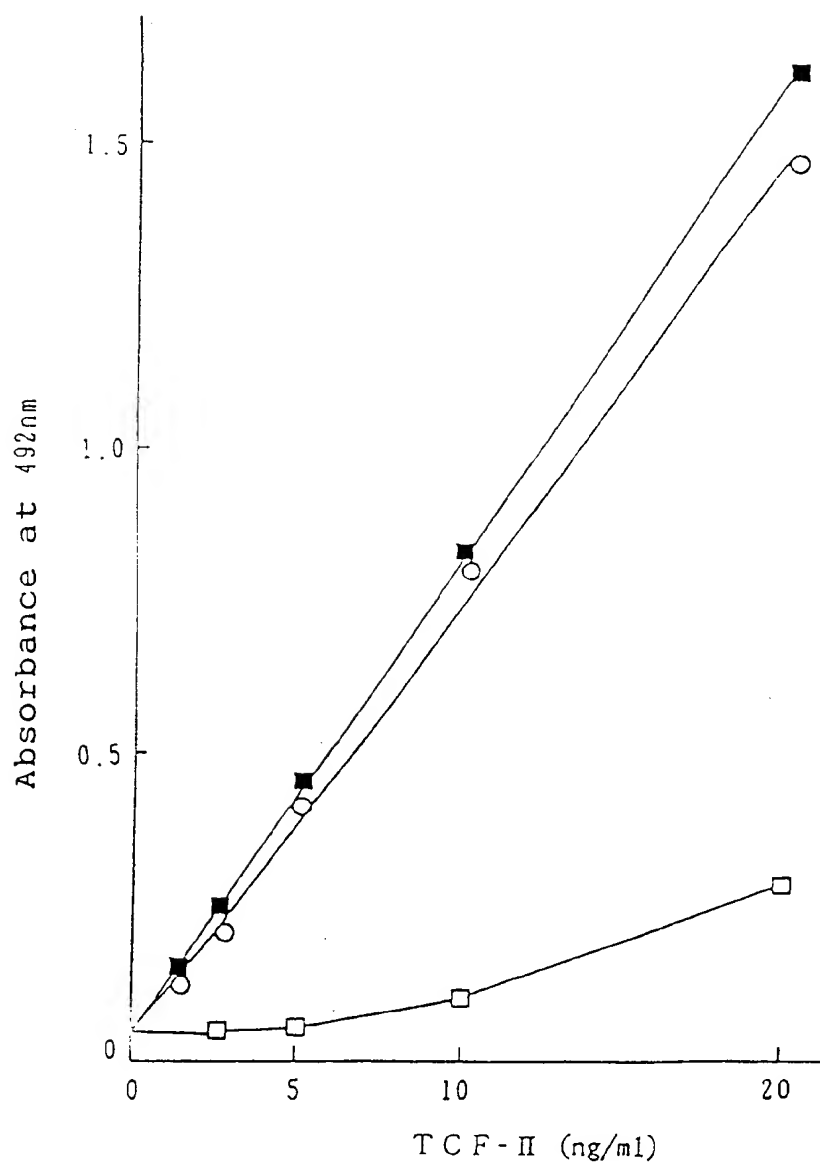


Fig. 2

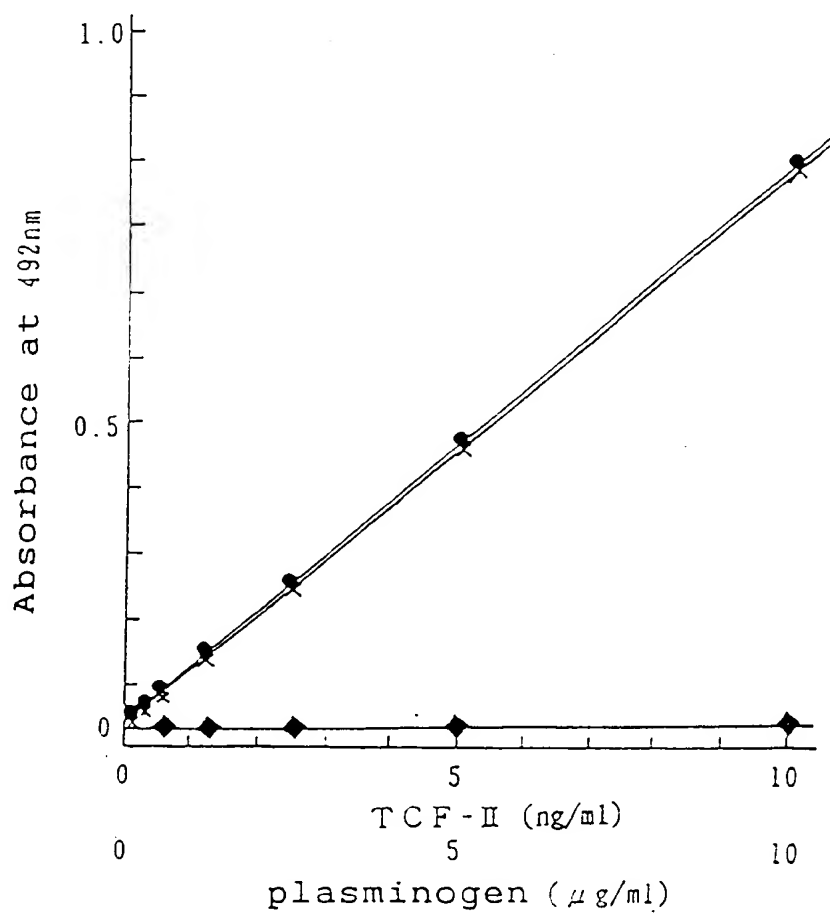


Fig. 3

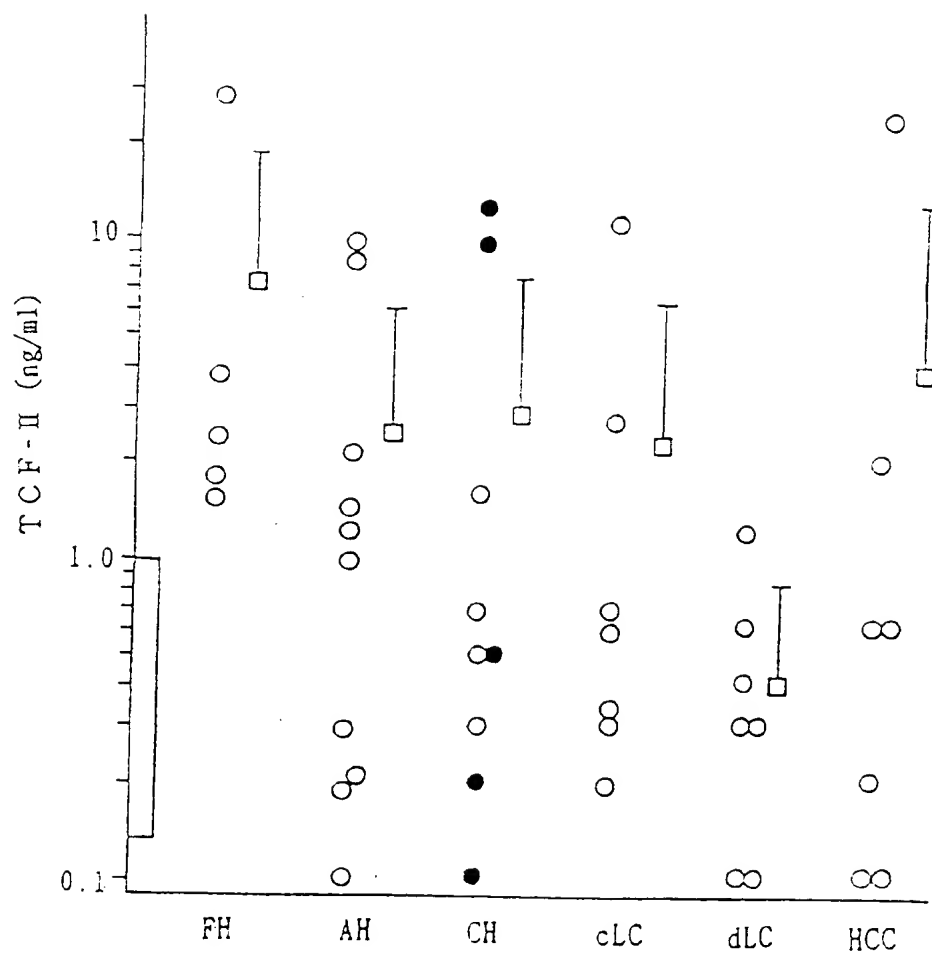




Fig. 4

